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13. ABSTRACT *(Maximum 200 words)*

Growth factors may play an important role in the pathogenesis of breast cancer. The MAP kinase pathway may be essential for transformation caused growth factors. Thus, the purpose of this study is to determine if MAP kinases may be overexpressed or activated in breast cancer. About one-third of the breast cancer cell lines tested contained changes in the amounts of MAP kinases, although there was not one consistent pattern of alteration from the normal expression. An alteration in amount of any of the enzymes may be an indication of altered regulation of the pathway in the affected cells. Future experiments to further address this issue will focus on analyzing activation state and correlating changes in amounts of MAP kinases with stage of tumor. Newly available antibodies allow measurement of activated MAP kinases directly in cell lysates. These antibodies are being evaluated for use in immunocytochemistry of normal mammary epithelial cells and breast cancer cell lines. If these antibodies prove useful for immunocytochemistry, in the future tumors will be obtained from Dr. Gazdar of this institution so that activation state of MAP kinases can be measured in situ.

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(5) INTRODUCTION

Breast cancer is the most common cancer of women (1). Although therapies have been developed that are effective in selected subgroups of patients, these therapies have not had a great impact on breast cancer mortality rates. To develop new and more effective therapies for breast cancer it will be important to understand the pathogenesis of breast cancer in more detail. Breast cancer cells express tyrosine kinase receptors and may be induced to proliferate by their cognate growth factors (1). The role of growth factors in human breast cancer is further supported by the finding that the *erb*B2 gene is amplified in 10-30% of breast cancers (2-10). The presence of this amplified gene correlates with a poor prognosis (2-10). Overexpression of ErbB2 in NIH 3T3 cells induces transformation, and expression of a mutant ErbB2 in transgenic mice results in development of adenocarcinomas of the breast in 100% of females (11-13). These data strongly support the conclusion that breast carcinoma cells are responsive to growth factors and support the hypothesis that these factors may play an important role in the pathogenesis of breast cancer.

Ras is an important signal transducer in the actions of tyrosine kinase receptors like those for ErbB2 and insulin (14). Activation of Ras leads to the activation of several downstream signaling pathways. Among these the best understood is the MAP kinase pathway (15). The GTP-bound form of Ras interacts with the protein kinase Raf, which, like Ras, is a proto-oncogene. The binding of Raf to Ras is thought to target Raf to the membrane where its protein kinase activity is increased. The enzymes activated by Raf, MAP kinase kinases or MEKs 1 and 2 (16), phosphorylate and activate the MAP kinases, ERK1 and ERK2. The MAP kinases ERK1 and ERK2 are pleiotropic regulatory enzymes activated in most if not all cell types by several of numerous hormones and growth factors. They phosphorylate many substrates, including certain other protein kinases and transcription factors, and have important growth regulatory functions. This pathway has also been implicated in the transforming activity of small t antigen (17).

Interfering with the functions of ERK1 and ERK2, using catalytically defective mutants of them, blocks 1) the actions of the oncogenic forms of Ras (18) and Raf in fibroblasts, 2) the transforming potency of small t antigen in CV-1 cells (17), and 3) cell proliferation induced by EGF (17). Constitutively active forms of the MAP kinases have not been identified either by genetic selection or by mutagenesis strategies (19). However, since the original proposal was submitted, it has been shown that activated mutants of MEK1 transform fibroblasts in culture, cause their growth in soft agar, and result in the formation of tumors in nude mice (20). MEK1 is one of the major targets of Raf, but it is probably not the only Raf target; thus, transformation induced by activated MEK1 is likely due to a subset of the potential actions of Raf. As the MAP kinases are the only known substrates of MEK1, it is believed that activated MEK1 transforms by activating MAP kinases. These findings support the concept that activation of MAP kinase leads to cellular transformation. Therefore, we propose that this pathway may be altered in breast cancer, perhaps increasing sensitivity to growth factors and that the activity of this pathway may be critical for the proliferation of the cells.

Given these critical role of tyrosine kinase receptors in breast cancer and the recent results with activated MEK1, the original purpose of this research was to: 1) measure the amounts of MAP kinases and MEKs in normal breast tissue and a series of cells from malignant breast; 2) measure the sensitivity of MAP kinases in these cells to insulin, serum, and EGF; 3) determine if activated MEK1 will transform cells from normal breast tissue; and 4) determine if catalytically defective mutants of MAP kinases block proliferation of breast cancer cells. The methods, described in more detail below, are primarily immunoblotting and activity assays developed in our laboratories, and morphological transformation assays. Newly available antibodies, described below, have caused us to consider amending our original goals to include immunocytochemistry of freshly isolated tumors. If these antibodies prove useful for immunocytochemistry, in the future tumors will be obtained from Dr. Gazdar of this institution for this analysis.

(6) BODY

Methodology

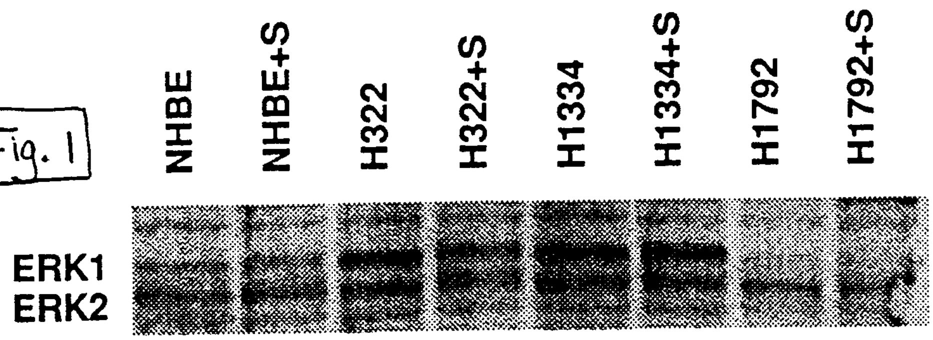
Immunodetection of enzymes of the MAP kinase pathway. The amounts of the MAP kinases present in the normal and malignant cells have been identified by immunoblotting with antibodies to ERK1 and ERK2 (21). We have generated antibodies to both isoforms that will detect both proteins cleanly in whole cell lysates by immunoblotting. ERK1 and ERK2 each undergo a shift in electrophoretic mobility when they are in the active forms. Thus, a lysate containing only inactive ERK1 and ERK2 will display two immunoreactive species, ERK2 at ~41 kDa and ERK1 at ~43 kDa. If the proteins are both fully active, two bands at ~42 kDa and ~44 kDa will be present. In a lysate containing a mixture of active and inactive ERKs, all four bands will be present and resolved. The differences in mobility are easily detected by comparison to a standard (we use lysates from PC12 or Rat 1 cells stimulated with NGF or insulin) containing all four forms.

Measurement of MAP kinase activity. A crude but reliable assay for stimulation of MAP kinase involves measurement of kinase activity in cell lysates using myelin basic protein (MBP) as the substrate. In the past this has been our initial screen, but we are now using immunoblotting to measure gel shifts as discussed above and using antibodies, now commercially available, that selectively recognize the active forms of ERK1 and ERK2. Because these antibodies do not recognize 100-fold excess of the inactive kinases, they can reliably detect even very small amounts of activated ERK1 and ERK2 in whole cell lysates and we are testing them for immunocytochemistry. If the methodology can be developed, immunocytochemistry will be performed on freshly isolated tumors to determine the activation state of the MAP kinases in situ.

Results

Examination of the MAP kinase pathway in cells from nonsmall cell lung carcinomas (nSCLC). In collaboration with John Minna's group ten nSCLC cell lines have been studied to probe the possible role of the MAP kinase pathway in this type of cancer. Some cell lines express mutated forms of Ras. The surprising finding was that several of these lines express much more of the MAP kinase ERK1 than normal bronchial epithelium (see H322 and H1334 in **Fig. 1**). To our knowledge this is the first finding of an elevated amount of a MAP kinase isoform in any human tumor. Previous efforts by us to identify altered amounts of MAP kinases in colon tumors indicated no change in ERK1 or ERK2 in the primary tumor or the surrounding tissue.

Fig. 1



Differences in the capacity of growth factors to activate the MAP kinase pathway in nSCLC cells were also noted. MAP kinase activity in the normal bronchial epithelial cells was only modestly sensitive to serum and unresponsive to insulin. In contrast, the cells that express more ERK1 have acquired insulin-sensitive MAP kinase activity, although they remain relatively insensitive to serum stimulation. These pilot studies performed on nSCLC cells are extremely promising in that they provide strong evidence that the MAP kinase pathway may contribute to the nSCLC phenotype. Additional studies by Minna's group examined possible mutations in MEK1 and MEK2 in nSCLC cells by RFLP. A mutation in MEK1 was found in one of 31 nSCLC lines tested but none were tumor cell specific; none were found in MEK2, although it was highly polymorphic.

Examination of the MAP kinase pathway in cells from human breast tumors and normal mammary epithelium. We have now examined MAP kinase amounts in a number of nonimmortalized lines from normal breast and breast carcinoma and lines from normal and malignant tissue that have been immortalized in the laboratory. Unlike normal bronchial epithelium, normal breast tissue has nearly equal amounts of ERK1 and ERK2. About one-third of the breast cancer cells contained changes in the amounts of these MAP kinases. Again, unlike in nSCLC, there was not one consistent pattern of alteration from the normal expression of ERK1 and ERK2. In some lines there was relatively more ERK1 and in some there was relatively more ERK2. An alteration in amount of any of the enzymes may be an indication of altered regulation of the pathway in the affected cells. However, the marked variation makes it difficult to form an initial hypothesis about the role of the cascade in breast cancer. More experiments will need to be performed. These will focus on analyzing activation state and correlating changes in amounts of MAP kinases with stage of tumor. In addition, current efforts are focused on using the antibodies that recognize active forms of ERK1 and ERK2 for immunocytochemistry of freshly isolated tumors.

One possible outcome is that the kinases in the pathway are more highly activated by growth factors in the malignant cells or have an altered kinetic response to the factors, suggesting that the kinases are involved in enhanced growth factor sensitivity. Alternatively we may be able to detect no differences in the activities of these kinases in the normal and malignant cells or in situ. If this is the case, we cannot conclude that the pathway is not involved. The malignant cells may express more or alternative substrates for enzymes of the cascade that would not be measured by these assays. A need for activation of this cascade should be discovered by future experiments using dominant negative ERK mutants or activated MEK1 mutants.

(7) CONCLUSIONS

About one-third of the breast cancer cells contained changes in the amounts of MAP kinases, although there was not one consistent pattern of alteration from the normal expression. An alteration in amount of any of the enzymes may be an indication of altered regulation of the pathway in the affected cells. However, the marked variation makes it difficult to form an initial hypothesis about the role of the cascade in breast cancer. Future experiments to further address this issue will focus on analyzing activation state and correlating changes in amounts of MAP kinases with stage of tumor. As discussed above newly available antibodies have caused us to consider amending our original goals to include immunocytochemistry of freshly isolated tumors. If these antibodies prove useful for immunocytochemistry, in the future tumors will be obtained from Dr. Gazdar of this institution for this analysis.

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